What is Life Like Inside a Cell? Probing the Metabolon with NMR

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Not until the work of Eduard Buchner in the late 19th century was it understood that biochemical reactions such as fermentation of sugars could proceed in cell-free extracts; he was awarded the 1907 Nobel Prize in Chemistry for this work (1). His discoveries, and developments by many others in methods for purifying and assaying metabolites, led to a scientific boom in enzymology and metabolism that continues today. Hence, the majority of the synthetic and degradative processes in cellular systems have now been mapped in great chemical detail. What is not well understood, even today, is the regulation of all these reactions and the inter-relationships of the fluxes in the multiplicity of enzymic pathways in whole cells and tissues.

While cells were being split open to extract metabolites and enzymes, questions were raised about the relevance of all the work on isolated enzymes and whether they behaved *in vivo* the way they do *in vitro*. So biochemists craved for a means of monitoring chemical and transport reactions in totally intact cells. A landmark experiment in this respect was reported by Moon and Richards in 1973 (2); they showed how ^{31}P NMR spectroscopy could be used to measure the concentration of ATP and 2,3-bisphosphoglycerate (2,3-BPG) in a suspension of intact rabbit red blood cells (RBCs). And, changes in the concentrations could be recorded over periods of hours from a single 3 mL sample without further intervention. Furthermore, it was observed that the spectral frequencies of 2,3-BPG and inorganic phosphate (Pi) changed in a systematic way that could be calibrated and related to pH inside the cell. Others quickly noticed this remarkable feature and within a year similar experiments had been conducted on skeletal muscle. In this latter work, use was made of the changes in frequency of the signal from Pi to measure non-invasively the pH inside muscle cells (3).

This was the NMR fever that heated my wing of the Biochemistry building when I began my postdoc as a Nuffield Demonstrator in Oxford in May 1975. I had gone there to work in the laboratory of the noted enzyme kineticist Keith Dalziel FRS. I continued developing computer models of metabolism and simulating enzymic reactions in the pre-steady-state regime, much as I had done during my PhD work in the Department of Physical Biochemistry, JCSMR, ANU. However, I longed for experimental approaches that would provide a check on the reality of these models of enzymic reactions *in situ*, in the proteinaceous 'soup' inside cells.

The NMR phenomenon had been discovered in 1945 and it was first used in understanding the magnetic properties of nuclei; it was initially considered to only be of interest to physicists but the discovery of chemical shift led chemists to exploit it in structure determination, especially in organic chemistry.

I became aware that while ^{31}P NMR provided a great leap forward in the understanding of muscle bioenergetics *in vivo*, much of metabolism was simply not seen through this new 'molecular microscope'. If only NMR could be used to study ^{1}H-containing compounds! After all, of the stable nuclides ^{1}H is the most sensitive to detection by NMR and it is found in virtually all biomolecules. But therein lies the problem! The water protons (~80 M) and non-exchangeable protons on proteins, that in RBCs amount to ~20 M, ensure that spectral peaks from low molecular weight solutes are totally obscured. It was thought in 1975 that ^{1}H NMR would only ever be useful for studying water transport and certain characteristics of the cell's interior like its viscosity, but it would never be useful for metabolic studies.

All that changed when my lab-bench-sharing friend Frank Brown explained his manipulation of the unwanted strong signals to enhance the peaks of the histidyl resonances from solutions of haemoglobin (Hb). My knowledge of computer models of RBC metabolism and metabolic defects of the RBC aroused my interest in this work. Together with an NMR physicist in the Biochemistry Department, Iain Campbell, Frank had shown how the resonances from the histidyl protons of Hb could be greatly enhanced by saturating the water spin-populations with long pulses of selective radio frequency radiation; and by combining this with the spin-echo technique, they obtained high-resolution spectra of the mobile moieties of Hb. In retrospect it seems like such a simple step to take, to place whole RBCs in the NMR spectrometer and 'see what could be seen'.

But it took Dallas Rabenstein on sabbatical leave from Edmonton in Canada, an expert in redox chemistry and knowledgeable in glutathione metabolism in RBCs, to suggest that it might be an informative experiment. I recall taking blood from Dallas by venipuncture and we washed the cells in physiological saline, and constituted in D_2O to help reduce the $^{1}H_2O$ signal, by repeated centrifugation and aspiration of the supernatant. The 0.5 mL sample of D_2O-washed RBCs was placed in the Bruker 270 MHz spectrometer… and the result was exhilarating; over a few minutes the peaks due to glucose declined before our eyes as the methyl resonance from lactate rose. We were observing metabolism in real time. This meant no more tedious extractions to measure glucose consumption and lactate production. Other metabolic studies involving glutathione oxidation and re-reduction followed in the succeeding days and our first paper on metabolic ^{1}H NMR was in press in *FEBS Letters* approximately six weeks later (4). Thus in 1977, our ^{1}H spin-echo NMR studies showed that reactions involving metabolites in the mM concentration range in whole RBCs could be studied non-invasively, on the 1-minute time scale.

It is interesting to note that the '270' in Oxford was the first commercial NMR spectrometer with a superconducting magnet to be placed in a university or industrial laboratory. And Professor Rex Richards, who

was the Dr Lees Professor of Physical Chemistry at Oxford, had decreed that it be located in the Biochemistry Department to facilitate the exploitation of its greatly enhanced sensitivity in the study of biological systems.

This paved the way for other applications with even higher field NMR spectrometers in studies of metabolism in many different cell-types and tissues and the exploitation of many new phenomena that would aid the quest for a better understanding of the processes that occur inside cells (eg 5,6). These suites of experiments provide some of the building blocks for contemporary *in vivo* studies in the human, especially the brain.

The most recent area of metabolic NMR to capture the imagination of the pharmaceutical industry and clinicians is metabolomics (also referred to as metabonomics). In this approach a ^{1}H NMR fingerprint is recorded from a biological fluid, such as blood plasma, urine, or cerebrospinal fluid; and the spectral pattern is correlated with the clinical state of the donor. NMR does not stand alone in this analysis, as mass spectrometry (MS) and ultra-high pressure liquid chromatography add complementary data to the sophisticated statistical analyses that yield diagnostic fingerprints (e.g. 7). Some MS analyses involve over 800 metabolites.

When I took up the offer made by Geoffrey Kellerman in 1977 to come and help set up the new Medical School at Newcastle, NSW, I did not know what lay ahead on the Australian NMR scene. I was soon pleasantly surprised. A few years previously the Australian Government had funded, for ~$5M, the National NMR Centre at the ANU. Its centrepiece was a Bruker HFX270 NMR spectrometer; its director was Alan J. Jones. Geoffrey had, typically, anticipated my research aspirations and lined up an appointment with Alan. Collaboration began almost immediately and Alan became an enthusiastic convert from applications of NMR to determine the structures of organic molecules to the use of the spin-echo to study RBC metabolism!

In Australia there are now over 60 NMR spectrometers with superconducting magnets, such is the interest in NMR analysis of samples from all walks of science, but especially biology. And the major driving force for the high-end spectrometers like the 800 MHz instruments at the ANU and WEHI is the determination of structures of proteins in solution. These instruments provide the much needed increase in sensitivity for metabolic / metabolomic studies as well. On the other hand, it is worth noting that there are well over one hundred 800 and 900 MHz spectrometers worldwide, so at present Australia lags behind the rest of the world in this technology; but moves are afoot to remedy this unacceptable state of affairs!

I will not conclude my story before mentioning three other items. First, the ABS / ASBMB has been a major part of my group's scientific life. Many new NMR-based results have been first presented at the annual scientific meetings; and it has been a forum in which young students from my group have first encountered international scientists including some of the top NMRists such as George Radda and Iain Campbell, and in some notable cases they have made post-doctoral plans with them. Second, while the focus of our projects has been biochemical, we have maintained a keen interest in developing NMR technology, principally software applications, but not exclusively; for example, we developed hardware for pulsed magnetic field gradients for diffusion measurements, which to my knowledge was the first in a superconducting magnet. In this respect my long-term colleague Bob Chapman has been invaluable. While I risk offending key colleagues and former students by not mentioning their names I must single out Bill Bubb who has meticulously run the NMR facility in our School since 1985. Finally, along this NMR journey, I have supervised ~40 Honours students and 19 PhD students since coming to Sydney in 1980; five of the latter are now full professors (Zoltan Endre, Kiaran Kirk, Glenn King, Bill Price and Lindy Rae). So, who said 'NMR' denotes 'no more research'?! I contend that the future of NMR in Australia is (t)rosy indeed.

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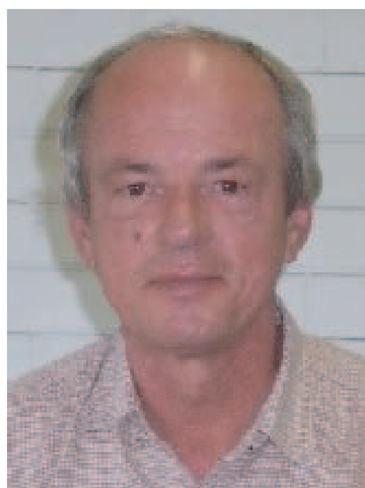

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